SHORT REPORT

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# Lack of Expression of *SERPINF1*, the Gene Coding for Pigment Epithelium-Derived Factor, Causes Progressively Deforming Osteogenesis Imperfecta With Normal Type I Collagen

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**ABSTRACT**

Osteogenesis imperfecta (OI) is a clinically heterogeneous heritable connective tissue disorder, characterized by low bone mass and reduced strength, which result in susceptibility to fracture and bone deformities. In most cases it is caused by dominant mutations in type I collagen genes, COL1A1 and COL1A2. Recessive forms, which collectively account for approximately 5% of cases of osteogenesis imperfecta detected in North America and Europe, are caused instead by mutations in various genes coding for proteins involved in collagen posttranslational modifications, folding, and secretion. A novel disease locus, *SERPINF1*, coding for pigment epithelium-derived factor (PEDF), has been found recently. In SERPINF1 mutants described so far, synthesis, posttranslational modification, and secretion of type I collagen were reported to be normal. Here we describe three siblings born to consanguineous parents, who show an initially mild and then progressively worsening form of OI with severe deformities of the long bones. They are homozygous for a frameshift mutation in exon 4 of the *SERPINF1* gene, which leads to lack of the transcription/translation product, likely a key factor in bone deposition and remodeling. Synthesis and secretion of type I collagen are normal. Clinical, radiographic, histological, and histomorphometric data from the proband are reminiscent of the distinctive features of type VI OI. 

**KEY WORDS:** BONE HISTOMORPHOMETRY; OSTEOGENESIS IMPERFECTA; PEDF; TYPE I COLLAGEN

## Introduction

Osteogenesis imperfecta (OI) is a heterogeneous heritable disorder of connective tissue characterized by reduced bone mass, bone fragility, and short stature. The majority of OI cases are due to dominant defects in either of the type I collagen genes, COL1A1 and COL1A2.

Mutations in these genes may cause different clinical phenotypes, ranging from perinatal lethal to very mild, comprised in the Sillence classification (type I to IV OI).[1] In a minority of OI patients the disorder is inherited in an autosomal recessive manner. Mutations in various genes have been recently identified in either homozygous or compound heterozygous patients affected by moderate to severe/lethal OI forms. The original Sillence classification is thus expanding in order to comprise additional types. Type VII OI (OMIM #610682), Type VIII OI (OMIM #610915), and Type IX OI (OMIM #259440) have been shown to be caused by defects in the genes encoding the components of the endoplasmic reticulum (ER)-resident collagen prolyl 3-hydroxylation complex: CRTAP, P3H1, and CyPB, respectively.[2–4] In a subset of recessive OI patients fibroblast collagen chains are normal. In this subset, mutations in genes

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such as FKBP10 and SERPINH1, coding for collagen chaperones FKBP65 and HSP47, respectively, resident in the ER, have been found.[5,6]

Two additional disease loci that are not directly involved in type I collagen production/secretion have also been identified. A loss-of-function homozygous mutation in SP7/OSX, the gene coding for the osteoblast specific transcription factor *Osterix*, caused severe OI in a young patient, born to consanguineous parents.[7]

Finally, exome sequencing in a few cases with a uniform diagnosis of type VI OI (OMIM #610968)[8] led to the identification of truncating mutations in SERPINF1, the gene encoding pigment epithelium-derived factor (PEDF), a secreted glycoprotein, was expressed in several tissues including osteoblasts.[9,10]

We describe here three siblings born to consanguineous parents, who have a progressively deforming type of OI that is associated with normal type I collagen production. The phenotype, characterized by grayish sclerae, normal dentition, and severe deformity of the long bones, is associated to a homozygous null allele mutation in exon 4 of the SERPINF1 gene.

## Patients and Methods

Cell cultures from skin biopsies and collagen screening were performed as detailed in the Supplemental Data.

### PEDF protein detection

Serum samples were obtained from two of the affected siblings (V-3 and V-4) and their mother (IV-2); conditioned fibroblast cultures media of patient V-1 were collected. Diluted sera, conditioned media, and cell layers were loaded on 10% polyacrylamide gels, fractionated by SDS-PAGE, electroblotted, and probed in Western blot by standard procedures, using a polyclonal antibody against human PEDF (Abcam, Cambridge, UK). Reactive bands were revealed by chemiluminescence with horseradish peroxidase (HRP)-conjugated secondary antibody (R&D Systems, Minneapolis, MN, USA). Amido black staining after protein transfer (serum and medium samples) and immunostaining with anti $\alpha$-tubulin (cell layer samples) were used as loading controls.

### Genetic and expression analysis

Coding regions and relative intronic boundaries of the SERPINF1 gene (MIM 172860; NM_002615.5) were amplified by eight specific intronic couples of primers.

Automated sequencing was performed on proband and parental DNA by a CEQ8800 capillary sequencer (Beckman Coulter, Indianapolis, IN, USA). denaturing high-performance liquid chromatography (DHPLC) (Transgenomics, Omaha, NE, USA) profiles allowed genotype determination for other family members.

The expression of the *SERPINF1* gene in patient V-1 fibroblasts was examined by qualitative reverse-transcriptase polymerase chain reaction (RT-PCR) assay. All primers and reaction conditions are available upon request.

### Histomorphometry

We fixed the bone sample in 70% ethanol and embedded it undecalcified in methyl-methacrylate resin. Sections were stained with Goldner's stain and measurements were performed by means of an image analysis system (Bone 3.5; Explora Nova, La Rochelle, France). Histomorphometric parameters were reported in accordance with the ASBMR Committee nomenclature. Data with fluorescence microscopy could not be obtained, because the patient did not undergo the tetracycline labeling treatment before biopsy specimen collection.

## Results

### Case report

The patients are siblings with progressively deforming moderate-severe OI. They were born to consanguineous Italian-Swedish and Italian-French healthy parents (first cousins and a half) residing in Switzerland (Fig. 1*A*).

Patient V-1 is a girl, 10 years 6 months old, wheelchair bound since the age of 6 years because of recurrent fractures; at present she can walk in the swimming pool. She was born at term after an uneventful pregnancy (birth weight [BW] = 3500 g, length [L] = 51 cm). At birth she had neither fractures nor limb deformities; she started walking around 12 months, she suffered her first fracture at 11 months of age (clavicle), at age 30 months long bone fractures became frequent ($\geq$30). The second affected girl (patient V-3) is 6 years 8 months old. She was born at term after an uneventful pregnancy (BW = 3600 g, L = 52 cm); at birth no limb deformities or other abnormalities were noted. Parents reported that she has suffered multiple fractures ($\geq$20) at lower limbs since the age of 6 months with consequent mild delay in gross motor development: she never started walking but she is currently training in order to stand with support. The younger patient (V-4) is 3 years 8 months old. She was born at term after an uneventful pregnancy (BW = 3800 g, L = 54 cm, cranium circumference [CC] = 34 cm); she has undergone 4 fractures since the age of 12 months, she started walking at 11 months, and she is still walking autonomously. All the girls have grayish sclerae and normal dentition, they do not show any particular dysmorphisms; no abnormalities in skin appearance and extensibility, no other health problems have been highlighted; in particular their vision is regular (without any retinal alteration) as well as liver, kidney, and pancreas functions and morphology. They show deformities of the long bones increasing in severity with age and moderate axial growth deficiency. Patient V-1 has evident deformities in upper and lower limbs; patient V-3 has severe arm deformities; the youngest sister (V-4) does not yet show any deformities. Their hand length and segmental proportions are appropriate for their age. On physical examination generalized, moderate ligamentous laxity and flattened occiputs were observed. Their intellectual development is normal. Biological test results (ie, routine blood cell count, blood and urinary levels of calcium, phosphate, creatinine, serum alkaline phosphatase, 25-hydroxyvitamin D, parathyroid hormone, osteocalcin, insulin-like growth factor 1 [IGF-1], and urine analysis) appear within normal ranges, whereas bone serum alkaline phosphatase and urinary excretion of the bone

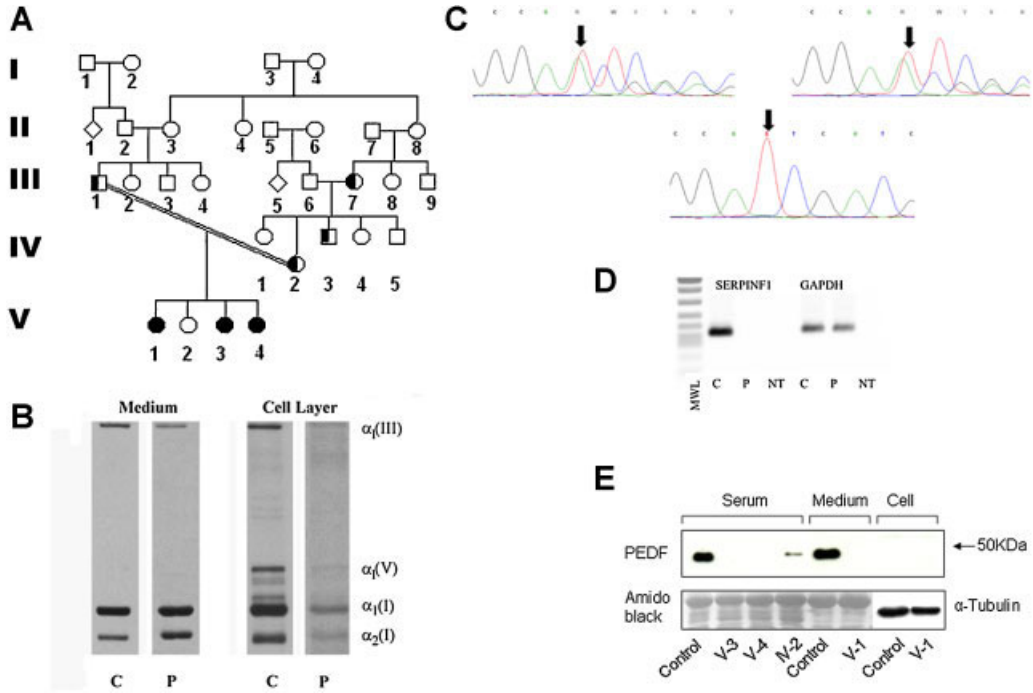


**Fig. 1.** Genetic and biochemical analysis. (*A*) Pedigree. Half-filled symbols indicate ascertained heterozygous carriers. Black symbols indicate OI-affected individuals. OI = osteogenesis imperfecta. (*B*) [$^3$H]-proline labeled collagens from control (C) and proband's (P) fibroblasts were purified either from medium or cell layer, pepsin-digested and run on 6% SDS-PAGE. (*C*) Upper row: black arrows point to heterozygous c.423delG mutation in parents (III-1 and IV-2); lower row: the black arrow points to homozygous c.423delG mutation in proband (V-3). (*D*) RT-PCR products of control (C) and proband (P) cytoplasmic mRNA. NT = no template; MWL = DNA molecular weight marker VIII (Roche Diagnostics GmbH, Mannheim, Germany). The reaction was run for either 30 cycles (SERPINF1) or 21 cycles (GAPDH). (*E*) Western blot of serum and dermal fibroblasts PEDF of the affected siblings, their mother and a healthy control. Top: PEDF signal and MW marker; bottom: amido black and α-tubulin loading controls.

resorption marker type I collagen telopeptide are slightly above the normal values; the radiographs show generalized osteopenia, with thin cortices, normal diaphyseal modeling in the long bones and no rickets signs (Fig. 2*D-G*) The vertebrae have multiple compressions and deformities. The lumbar dual-energy X-ray absorptiometry (DXA), obtained using Hologic, Inc. (Bedford MA) bone densitometer showed values lower than the third percentile (0.296 g/cm$^2$) in patient V-3, at age 6 years 7 months. A transiliac bone biopsy was obtained from patient V-3, during an urgent surgery session, upon informed parental consent.

### Collagen screening

SDS-PAGE analysis of radiolabeled collagens did not show any abnormalities; collagen chains were fully secreted into the medium and the relative ratio with type III collagen was in the normal range (Fig. 1*B*).

### Identification of the causal mutation

Sequence analysis revealed a homozygous c.423delG mutation in SERPINF1 exon 4 in the proband, both parents are heterozygous (Fig. 1*C*). Homozygosity for the mutation was confirmed in the two affected sisters, while it was not found in the unaffected sister (V-2) (data not shown). Screening of other candidate genes (see Supplemental Data) had given negative results. The deletion shifts the reading frame and introduces a premature stop codon 26 nucleotides downstream within the coding sequence, inside exon 5 (p.Ile142Ser fs*9). RT-PCR evaluation of SERPINF1 mRNA in the proband's fibroblasts gave negative results (Fig. 1*D*); further quantitation by real-time RT-PCR revealed barely detectable levels (1.4% of control) of gene expression (Supplemental Fig. 1).

### PEDF protein detection

Western blot analysis in serum (patients V-3 and V-4) and in cultured fibroblasts (patient V-1), was unable to detect PEDF protein; a faint band was detectable instead in their heterozygous mother's serum (IV-2) (Fig. 1*E*).

### Bone histology

The histomorphometric results are reported in Table 1. The histological pattern revealed increased osteoid thickness, surface, and volume, compared with age-matched control samples.[11] In addition, we observed increased osteoclast activity (eroded surface/bone volume [ES/BV] and osteoclast number [OcN]). Qualitative evaluation showed loss of the normal orientation of the lamellae under polarized light, showing areas reminiscent of the "fish-scale" pattern described by Glorieux and colleagues[8] (Fig. 2).

## Discussion

Three sisters affected by moderately severe, progressively deforming IO were studied. A noteworthy observation is that

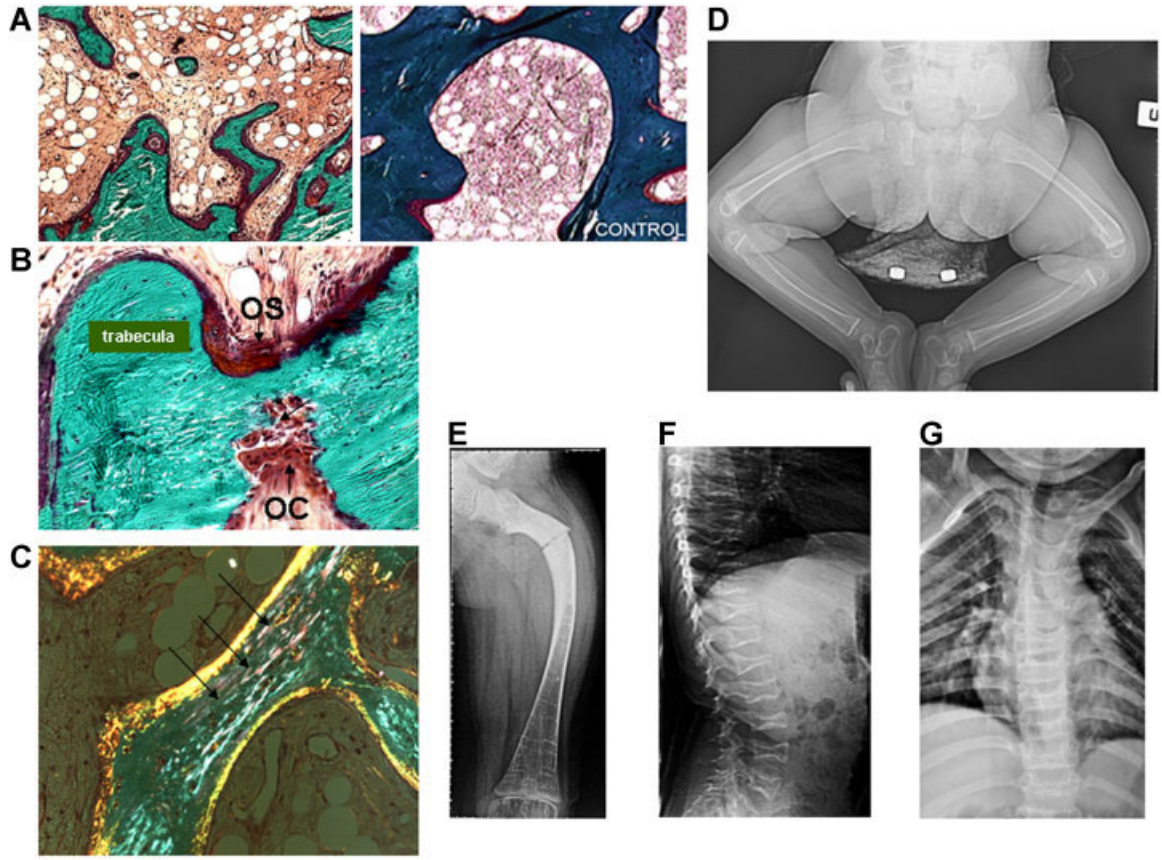


**Fig. 2.** Histological and radiological data. (*A*) Glodner-stained bone section from the patient. Resorption lacunae and a large quantity of unmineralized osteoid (in red) are visible, indicative of a mineralization defect. Control: iliac bone section of an age-matched control with normal amount of osteoid. (*B*) Bone resorption can be appreciated as well, with increased number and size of osteoclasts (OC, magnification ×200). (*C*) Bone section under polarized light. Arrow points to anomalies in the orientation of lamellae, reminiscent of a "fish-scale" pattern (magnification ×200). (*D*) Legs of infant V3 at 6 months of age. Her long bones showed mild osteoporosis and thin cortices. (*E*) Age 6 years. Multiple fractures healing resulted in the lack of modeling and considerable bowing of lower limbs. ( *F*,*G*) lateral and frontal views of the spine. The vertebrae were severely osteoporotic with multiple compressions and deformities.

they appeared healthy at birth and did not suffer any fractures until 6 to 12 months of age. Routine screening of type I collagen chains synthesized by the proband's fibroblasts did not reveal any structural abnormality. Negative results from mutation screening in COL1A1 and COL1A2 genes drove us to consider additional disease genes associated to recessive OI forms. A homozygous deletion in exon 4 (c.423 delG) was found in the SERPINF1 coding sequence (NM_002615.5); it leads to a loss-of-function phenotype due to nonsense-mediated decay of mRNA.

**Table 1.** Histomorphometric Results

| | | Normal values[11] |
|---|---|---|
| Bone volume/tissue volume (%) | 14.6 | $17.7 \pm 2.6$ |
| Trabecular thickness (μm) | 79 | $101 \pm 11$ |
| Osteoid thickness (μm) | 14.4 | $5.8 \pm 1.4$ |
| Osteoid surface/bone surface (%) | 26.0 | $34.0 \pm 6.7$ |
| Osteoid volume/bone volume (%) | 16.00 | $3.97 \pm 1.19$ |
| Erosion surface/bone surface (%) | 21.2 | $14.8 \pm 4.4$ |
| Osteoclast surface/bone surface (%) | 3.60 | $1.11 \pm 0.75$ |

All thickness/depth results are corrected for obliquity of sections by multiplying by $\pi/4$.

Heterozygous carriers of the SERPINF1 null mutation in the family have, as expected, lower-than-normal levels of serum PEDF; they are healthy with normal stature and did not report skeletal problems or bone fractures.

Lack of the SERPINF1 gene product, PEDF, was associated for the first time with severe OI in two unrelated patients.[9] More recently two additional loss-of-function mutations were identified in three patients previously diagnosed as type VI OI, whose clinical features are very similar to those described in our case report.[10]

PEDF, a 50-kDa secreted glycoprotein of 418 amino acid residues with high affinity to collagens of the extracellular matrix (ECM), has shown multiple and varied biological properties.[12–14] PEDF is believed to play a role in bone homeostasis as an inhibitor of bone resorption, because it upregulates osteoprotegerin, which inhibits osteoclast maturation.[15] Its strong anti-angiogenic activity interplays with the pro-angiogenic activity of vascular endothelial growth factor (VEGF); the balance between these two factors is probably crucial in the process of bone formation and resorption. In fact, PEDF is capable of inducing differentiation of precursor cells into mature osteoblasts, whereas VEGF facilitates osteoclast formation and bone resorption function. PEDF supposedly behaves as a functional

antagonist of VEGF because it can downregulate its expression.[16] In fibroblasts, which do not produce PEDF, we observed an increase of VEGFA gene expression; the patient's serum levels of VEGF are above the average of age-matched controls (see Supplemental Data). These preliminary data need further investigation. Lack of PEDF apparently has not so far affected other tissues and organs in our young patients. A thorough clinical follow-up is necessary nonetheless, considering the diverse physiological activities exerted by the protein in various tissues. Besides the already mentioned anti-angiogenic effects, PEDF also has anti-tumor activity, and neurotrophic and neuroprotective activities in the central nervous system; it is also highly expressed in the liver, where it exerts its effects on glucose and lipid metabolism.[17] Histological and histomorphometric data obtained from an iliac crest bone biopsy of the proband showed increased osteoid thickness, surface, and volume, and increased osteoclast activity, compared to reference values. The histological pattern of the patient's sample is comparable to those described previously for type VI OI patients, recently identified as SERPINF1 mutants. We suggest an explanation for our histological findings on the basis of the elucidated molecular defect. The presence of unmineralized osteoid and the disorganization of the bone matrix, coupled with increased bone resorption parameters, may result from the impaired balance between PEDF and VEGF; lack of PEDF entails both a defect in bone neoformation and the upregulation of VEGF levels, thus favoring osteoclastic activity. We are aware, nevertheless, that further investigation is needed to understand better why the absence of PEDF causes the OI type VI phenotype.

Other clinical features of OI type VI recur in the family here described: absence of dentinogenesis imperfecta, white/grayish sclerae, moderate ligamentous laxity, and severe progression of the disease. Investigations in other SERPINF1 mutants are presently ongoing and may contribute to a further definition of the peculiar clinical features of this OI form. Finally, considering our experience and published studies,[18] because bisphosphonates treatment appears to obtain poor results in type VI OI, we believe that for these patients strategies of replacement therapy warrant investigation, given that PEDF therapeutic potential and safety have been tested already for cancer therapy.[19]

## Disclosures

All authors state that they have no conflicts of interest.

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